

## OR BINDING KINETICS

pression of interleukin 2 receptors.  
1995.

J. L. Strominger. 1984. Antigenic  
skin 2 receptor on human T cells.

functional interleukin 2 receptors  
and.). 331:267.

T. Honjo. 1986. Molecular basis  
ceptor: affinity conversion model.

Z. Saito, H. Sabe, N. Matsunami,  
kin 2 Receptor: Structure, Func-  
Biol. 11:739.

A. Gray, A. W. Tom, J. Lee, Y.  
ird, E. L. V. Mayers, N. Whittle,  
epidermal growth factor receptor  
ied gene in A431 epidermoid car-

M. Rottman, L. B. Crittenden,  
in ALV-induced erythroblastosis:  
expression of an amino-truncated

N. Kobayashi, M. Hatanaka, N.  
Properties of human IL-2 receptors  
on. *Mol. Biol. Med.* 2:379.

monoclonal antibody (anti-Tac)  
in cells. I. Production of anti-Tac  
ls. *J. Immunol.* 126:1393.

and high affinity cellular receptors

*zymol.* 43:103.

Exhibit G

## CYTOKINES IN CHRONIC INFLAMMATORY ARTHRITIS

### I. Failure to Detect T Cell Lymphokines (Interleukin 2 and Interleukin 3) and Presence of Macrophage Colony-stimulating Factor (CSF-1) and a Novel Mast Cell Growth Factor in Rheumatoid Synovitis

By GARY S. FIRESTEIN, WEI-DONG XU, KAY TOWNSEND,\* DAVID BROIDE,  
JOSE ALVARO-GRACIA, ANDREW GLASEBROOK,\*  
AND NATHAN J. ZVAIFLER

From the Department of Medicine, Rheumatology Division, University of California, San Diego,  
San Diego, California 92103; and \*Lilly Research Laboratories, La Jolla, California 92037

Current models of rheumatoid arthritis (RA)<sup>1</sup> implicate lymphokines in the recruitment and activation of T cells in the synovium (1, 2). However, adequate documentation of significant amounts of these proteins in the joint is limited. In fact, recent studies have found only very low levels of IFN- $\gamma$  in synovial fluid (SF) and tissue of patients with RA (3). This relative lack of IFN- $\gamma$  seems contradictory in light of morphologic and immunohistochemical evidence of T cell activation in the RA synovium (4-6).

Because of this paradox, we decided to study two other T cell lymphokines (IL-2 and IL-3) in rheumatoid synovitis. In this study, we report that neither IL-3 RNA transcript nor IL-2 could be detected in rheumatoid joint samples. However, colony-stimulating factor (CSF) and mast cell growth factor (MCGF) were observed. These data support the notion that T cell lymphokines are not abundant in the rheumatoid joint and that most soluble inflammatory mediators appear to be produced by macrophages, synovial lining cells, fibroblasts, and endothelial cells in the synovium.

### Materials and Methods

**Cytokines.** Human (h) rIL-2, hCSF-1 (macrophage (M)-CSF), and the hCSF-1 cDNA were obtained from Cetus Corp. (Emeryville, CA). hrIL-3 and hrIL-4 were purchased from Genzyme (Cambridge, MA). Human recombinant granulocyte/macrophage (GM)-CSF and hrIFN- $\gamma$  were obtained from Amgen Biologicals (Thousand Oaks, CA). Murine (m) rIL-3, hG-CSF, and hIL-1 and the hIL-2-R cDNA were obtained from Lilly Research Laboratory (La Jolla, CA). Genetics Institute (Cambridge, MA) provided the human IL-3 cDNA. IFN- $\alpha_2$  (Gxa 01-901-535) was obtained from the National Institutes of Health, Bethesda, MD.

**Animals.** For CSF assays, 4-12-wk-old CBA/J mice were used. For MCGF assays, 4-12-wk-old BALB/c mice were used.

This work was supported in part by National Institutes of Health grants CA-09174, AR-14916, AI-00845, AI-20476, AI-17268, and AI-20194, and by an Arthritis Foundation Biomedical Research Center Grant. Address correspondence to Dr. Gary S. Firestein, University of California, San Diego, 225 Dickinson Street, H-811-G, San Diego, CA 92103.

<sup>1</sup> Abbreviations used in this paper: CSF, colony-stimulating factor; G, granulocyte; h, human; m, mouse; M, macrophage; MCGF, mast cell growth factor; RA, rheumatoid arthritis; SF, synovial fluid; ST, synovial tissue.

**Patients.** Materials from patients with active classic or definite RA (7) or other forms of chronic inflammatory synovitis, such as psoriatic arthritis, Reiter's syndrome, and ankylosing spondylitis, were studied. Joint aspirations were only performed if clinically indicated; informed consent was obtained before the procedure in accordance with Human Subjects Committee requirements.

**SF and ST Supernatant Preparations.** SF was aspirated aseptically into heparinized syringes from the knee joint of patients with active arthritis. The fluid was separated from cells by centrifugation at 400 g and frozen at  $-70^{\circ}\text{C}$  until assayed. ST supernatants were prepared as previously described (3). Briefly, rheumatoid tissue obtained at the time of total joint replacement surgery was digested for 3 h with collagenase and DNase (Sigma Chemical Co., St. Louis, MO) at  $37^{\circ}\text{C}$ . The resultant single cell suspension was washed and filtered through a nylon mesh and the red cells were lysed with hypotonic PBS. Cells were cultured at a density of  $2-3 \times 10^6/\text{ml}$  in RPMI 1640 supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY) for 3 d at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Supernatants were harvested and filtered after 3 d and frozen at  $-70^{\circ}\text{C}$  until assayed.

**IL-2 Bioassay.**  $4 \times 10^3$  CTLL cells (8) were cultured in microtiter wells in 100  $\mu\text{l}$  of DME (Gibco Laboratories) plus dilutions of the test sample for 28 h. [ $^3\text{H}$ ]TdR was added for the final 4 h of culture. Cells were harvested and thymidine uptake was determined in a scintillation counter. In some experiments, the mAb PC61 (9) was preincubated with the cells at  $37^{\circ}\text{C}$  for 30 min before the addition of test sample in order to block IL-2-R.

**IL-2 Immunoassay.** Microtiter wells were coated with 50  $\mu\text{l}$  of mAb to hIL-2 overnight at  $4^{\circ}\text{C}$ . The wells were then blocked with 5% casein for 18 h at  $4^{\circ}\text{C}$ . After plates were washed with PBS-Tween, 50  $\mu\text{l}$  of each sample was added to duplicate wells and incubated at  $37^{\circ}\text{C}$  for 2 h. After washing with PBS-Tween, rabbit anti-h IL-2 (Genzyme) diluted in 5% casein was incubated in the wells for 2 h at room temperature. Plates were again washed and goat anti-rabbit antibody conjugated to alkaline phosphatase was incubated in wells for 2-3 h at room temperature. After washing, 50  $\mu\text{l}$  of *p*-nitrophenyl phosphate (1 mg/ml) (Sigma Chemical Co.) was added. Plates were read on a micro-ELISA reader (Dynatech Laboratories, Inc., Chantilly, VA) at a wavelength of 405 nm. rIL-2 was used as a positive control.

**MCGF assay.** Bone marrow cells from BALB/c mice were grown for 3 wk in flasks containing RPMI 1640 plus 10% FCS and 20% WEHI-3 supernatant (containing mIL-3). Approximately 99% of viable cells were mast cells as shown by morphology and toluidine blue stain in acid. Mast cells were resuspended in RPMI 1640 plus 10% FCS and  $5 \times 10^4$  were cultured in quadruplicate wells at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 48 h. Cells were pulsed with [ $^3\text{H}$ ]TdR during the final 18 h, harvested, and counted on a scintillation counter.

**Murine CSF Assay.** Bone marrow cells were harvested from CBA/J mice and washed in DME plus 10% FCS. Adherent cells were removed by incubating the cells in plastic petri dishes for 1-2 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ .  $2.5 \times 10^5$  nonadherent cells were resuspended in 2.5 ml of modified Iscove's medium,  $10^{-3}$  M 2-ME, 1% methylcellulose, 25% FCS, and test factor. SF were tested at a 1:10 dilution and ST supernatants were tested at either a 1:10 or 1:5 dilution. 1 ml of the suspension was added to duplicate 35-mm petri dishes and cultured at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in a humidified incubator. Colonies ( $>50$  cells) were counted using an inverted microscope on day 10. In some experiments the cells were fixed to slides using a cytospin apparatus and were stained with Wright-Giemsa stain.

**Characterization of CSF and MCGF.** SF or ST supernatant was concentrated two- to fivefold and fractionated on a 75 cm  $\times$  1.5 cm Sephadex G-100 column at  $4^{\circ}\text{C}$ . Each 3-ml fraction was assayed for CSF and MCGF.

**Riboprobe Protection Assay.** Riboprobe protection assays were performed as previously described (10). Briefly, synovial cells were solubilized in 7.6 M guanidine hydrochloride (J. T. Baker Chemical Co., Phillipsburg, NJ), 100 mM potassium acetate (Sigma Chemical Co.), 0.5% *n*-lauroyl sarcosine (Sigma Chemical Co.), 100 mM 2-ME (Sigma Chemical Co.). RNA was precipitated by the addition of ethanol. RNA probes were prepared using linearized DNA template, cold nucleotides,  $\alpha$ -[ $^{32}\text{P}$ ]UTP (ICN Pharmaceuticals, Inc., Irvine, CA), and SP6 polymerase (Promega Biotech, Madison, WI). The RNA from  $10^6$  cells was hybridized with  $\alpha$ - $^{32}\text{P}$ -labeled RNA probes in 80% formamide overnight at  $42^{\circ}\text{C}$ . Unhybridized probe was then digested with ribonuclease A and incubated with proteinase K (both from Sigma Chem-

RA (7) or other forms of  
ndrome, and ankylosing  
clinically indicated: in  
Human Subjects Com-

into heparinized syringes  
separated from cells by  
ernatants were prepared  
ime of total joint replace-  
igma Chemical Co., St.  
ted and filtered through  
were cultured at a den-  
S (Gibco Laboratories,  
e harvested and filtered

wells in 100  $\mu$ l of DME  
Tdr was added for the  
etermined in a scintilla-  
ubated with the cells at  
ck IL-2-R.

Ab to hIL-2 overnight  
After plates were washed  
and incubated at 37°C  
e) diluted in 5% casein  
again washed and goat  
ated in wells for 2-3 h  
1 mg/ml) (Sigma Chem-  
tech Laboratories, Inc.,  
sitive control.

for 3 wk in flasks con-  
taining mL-3). Ap-  
logy and toluidine blue  
FCS and  $5 \times 10^4$  were  
Cells were pulsed with  
llation counter.

J mice and washed in  
he cells in plastic petri  
ls were resuspended in  
osc, 25% FCS, and test  
tested at either a 1:10  
m petri dishes and cul-  
ills) were counted using  
re fixed to slides using

intrated two- to fivefold  
°C. Each 3-ml fraction

formed as previously de-  
ne hydrochloride (J. T.  
(Sigma Chemical Co.),  
ia Chemical Co.). RNA  
d using linearized DNA  
, Irvine, CA), and SP6  
ills was hybridized with  
nhybridized probe was  
oth from Sigma Chem-

ical Co.). The protected fragments of probe were phenol/chloroform extracted, ethanol precipi-  
tated, and resolved on a 6% 20:1 acrylamide/bis-acrylamide sequencing gel. The gel was  
exposed at -20°C using Kodak XAR film and an intensifying screen.

**Radioimmunoassay.** A specific RIA for measuring CSF (CSF-I) was performed under the  
direction of Dr. Peter Ralph at Cetus Corp.

**Statistics.** Statistical analysis was performed using Student's *t* test. Data are presented as  
mean  $\pm$  SEM.

## Results

### Low Levels of IL-2 in RA SF

**Bioassay.** The IL-2-dependent murine cell line CTLL was incubated with var-  
ious concentrations of hrIL-2. As shown in Fig. 1 A, there was a saturable, dose-  
dependent increase cell proliferation, as assessed by [ $^3$ H]Tdr incorporation. When  
cells were preincubated with the mAb PC61 (which competitively blocks the IL-2-  
R), there was no effect in the presence of saturating amounts of IL-2. At lower con-  
centrations of IL-2, PC61 inhibited IL-2-induced CTLL proliferation. Using this  
method, 14 RA SF were assayed for IL-2 activity. Six (43%) induced a significant  
increase in thymidine uptake (greater than three times medium control). Preincu-  
bation of cells with PC61 had no effect on proliferation induced by any of the six  
positive samples (see Figs. 1, B and C, for representative experiments). Therefore,  
the CTLL-stimulating activity in SF was not IL-2.

**Immunoassay.** A specific ELISA for IL-2 was used to confirm the bioassay results.  
This assay can detect 25-50 pg/ml of hrIL-2 ( $3 \times 10^6$  U/mg). Using this method,  
the supernatants of PMA (Sigma Chemical Co.)-stimulated HUT cells (a human  
T cell line) were found to contain  $\sim 10$  ng/ml of IL-2. 15 RA SF samples were as-  
sayed using the ELISA (including 2 that were positive in the CTLL proliferation  
assay) and no IL-2 was detected.

### Growth Factor Activity in SF

When these experiments were initiated, testing for IL-3 was problematic because  
human IL-3 had not been identified and no reliable assay was available. In the mouse,  
however, it was known that IL-3 had many activities, including the ability to func-  
tion as a multilineage colony stimulating factor as well as to stimulate mast cells  
and IL-3-dependent leukemic cell lines (11). Extrapolating from studies in mice, two  
assays were used to study synovial fluids; namely a CSF assay using bone marrow  
of mice and a murine MCGF assay.

**Proliferation of Murine Bone Marrow Mast Cells.** A cell population containing >99%  
mast cells was prepared by cultivating murine bone marrow in the presence of WEHI-3  
supernatant (which is a rich source of mL-3) for 3 wk. These mast cells proliferated  
in response to mL-3 and WEHI-3 supernatant but not to hrIL-1 $\beta$ , IL-2, IL-4,  
CSF-I, GM-CSF, G-CSF, IFN- $\gamma$ , IFN- $\alpha$ , LPS, or PHA (data not shown). The results  
of experiments assaying joint samples for MCGF activity are summarized in Table  
I. As expected, the WEHI supernatant was a potent stimulator of mast cell prolifer-  
ation. A representative dose response is shown in Fig. 2 A. 20 RA samples were  
assayed (three representative dose responses are shown in Fig. 2 B). [ $^3$ H]Tdr up-  
take was significantly greater for RA SF ( $2,910 \pm 328$  cpm) compared with either  
plasma or medium controls ( $p < 0.001$ ). Of the 20 rheumatoid fluids tested, 8 fluids  
were studied with extensive serial dilutions. In 4 of these, proliferation was relatively

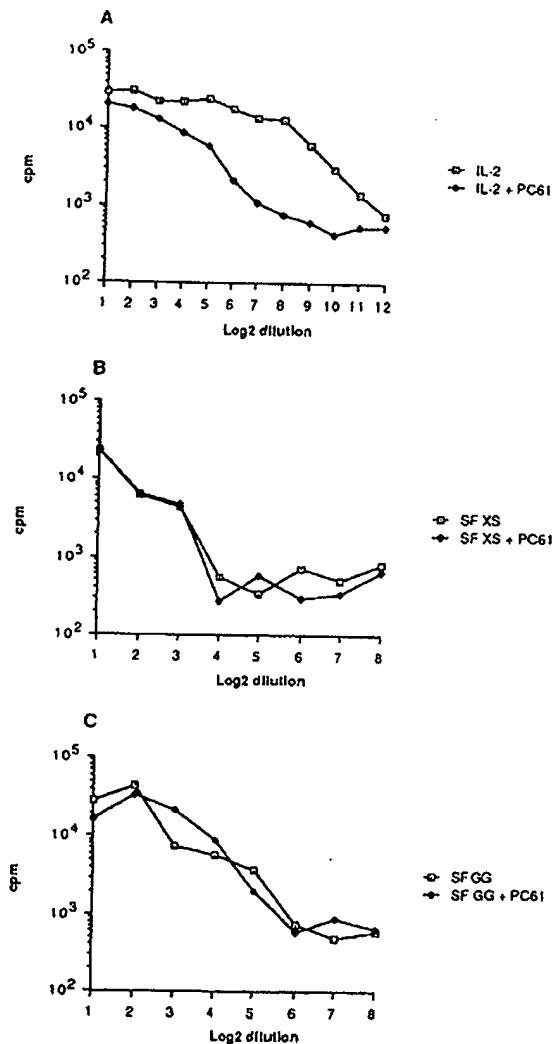


FIGURE 1. IL-2 assay. CTLL cells were cultured with serial dilutions of IL-2 or test sample for 28 h and pulsed with  $[^3H]ThR$  for the last 4 h. In some experiments, cells were preincubated with mAb PC61, which binds to the mIL-2-R and competes with both human and mIL-2. (A) Dose-response curve for IL-2 with and without PC61. (B and C) Representative examples of dose-response curves for (SF) that stimulated CTLL proliferation. Preincubation of cells with PC61 did not block proliferation.

low at high concentrations of SF and did not peak until at least a 1:16 dilution (see Fig. 2 B for two examples). This has two implications; First, the level of proliferation at low concentrations of SF approached that of WEHI supernatant, which suggests that substantial quantities of MCGF are present. Second, an inhibitor of MCGF interferes with the mast cell proliferation at high concentrations of SF.

Two supernatants of cultured RA ST were assayed. Both significantly increased mast cell proliferation (see Table I). The MCGF activity was not found exclusively in RA samples. Rheumatoid variant SF were also positive, but the level of prolifera-

TABLE I  
Mast Cell Growth Factor Assay

Sample	$[^3\text{H}]\text{TdR}$ uptake
	<i>cpm</i>
Medium ( $n = 7$ )	$493 \pm 67$
Normal plasma ( $n = 4$ )	$520 \pm 128$
WEHI 1:2 ( $n = 6$ )	$31,710 \pm 4,040$
WEHI 1:16 ( $n = 6$ )	$5,478 \pm 688$
WEHI 1:32 ( $n = 7$ )	$2,318 \pm 250$
RA SF ( $n = 20$ )	$2,910 \pm 329^*$
RA ST ( $n = 2$ )	$2,965$
RV <sup>5</sup> SF ( $n = 7$ )	$1,246 \pm 156^{\dagger}$
OA <sup>1</sup> SF ( $n = 3$ )	$736 \pm 100$

\*  $p < 0.001$  compared with medium and  $p < 0.05$  compared with RV SF.

$p < 0.001$  compared with medium.

<sup>§</sup> RV, rheumatoid variant (including psoriasis, Reiter's syndrome, and ankylosing spondylitis).

OA, osteoarthritis.

tion ( $1,246 \pm 156$  cpm) was less than that for RA SF ( $p < 0.05$ ). Noninflammatory osteoarthritis SF did not support mast cell proliferation.

**CSF Assay Using Murine Bone Marrow.** SF and ST were assayed for CSF activity on nonadherent bone marrow cells from CBA/J mice. After 10 d, colonies (>50 cells) were counted (see Table II). hrGM-CSF had no effect on murine marrow cell colony

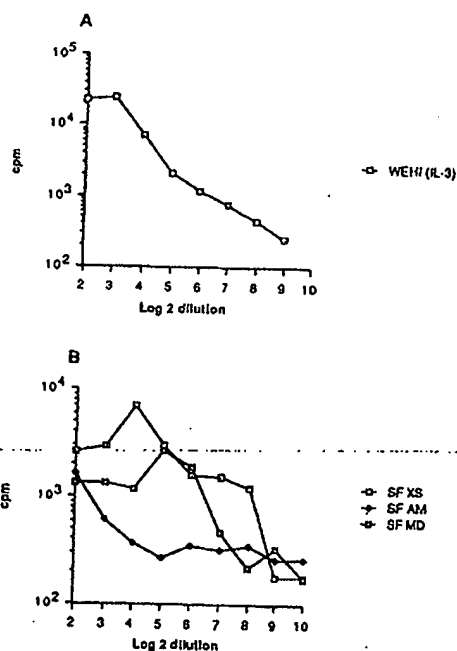


FIGURE 2. Mast cell proliferation assay. (A) Dose-response curve of WEHI-3 supernatant (IL-3) on murine mast cells. Murine bone marrow-derived mast cells (see text) were cultured for 48 h with serial twofold dilutions of WEHI supernatant or test sample. Cells were pulsed with [ $^3$ H]TdR for the last 18 h. (B) Representative examples of RA SF dose responses on murine mast cells.

at least a 1:16 dilution (see Fig. 1). At this level of proliferation, the supernatant, which suggests the presence of an inhibitor of MCGF proliferation, was found at concentrations of SF.

both significantly increased was not found exclusively, but the level of prolifera-

TABLE II  
Effect of ST and SF on Murine Bone Marrow

Sample	Colonies/10 <sup>5</sup> cells*
Medium	0
mIL-3 (100 U/ml)	110
hGM-CSF (80 U/ml)	0
hCSF-1 (50 U/ml)	34
RA SF (n = 5)	34 ± 24
RA ST (n = 5)	21 ± 5
Psoriatic SF (n = 3)	0
RA serum (n = 4)	0
Normal serum (n = 3)	0

\* Colonies counted after 10 d. Data expressed as mean number of colonies per 10<sup>5</sup> cells in duplicate cultures.

formation, although human G-CSF (data not shown) and CSF-1 were active. Five RA SF samples were tested in the CSF assay, and four were positive (see Table II). All five supernatants from RA ST supported colony growth. Three of the five RA SF and 2/5 RA ST had been previously tested for MCGF and all were positive. Colonies appeared between days 3 and 5 and persisted for >2 wk. Some disease specificity was suggested by the finding that none of the three psoriatic SF tested were positive. When individual colonies were selected from RA fluid cultures and stained, most were found to contain macrophages, although some mixed colonies containing both granulocytes and macrophages were occasionally identified. Because the factor was produced by ST explants, it would appear to be made locally. In support of this, we found that four RA and three normal serum samples were negative in the CSF assay despite the fact that one of these patients had high CSF activity in SF (120 colonies/10<sup>5</sup> cells).

**Characterization of CSF and MCGF Activity in RA SF.** While these studies were in progress, the hIL-3 gene was cloned (12) and the recombinant product was produced. To our surprise, hIL-3 did not stimulate either murine mast cells or murine bone marrow cells. This species specificity meant that the factors detected in the mast cell proliferation assay and the CSF assay were distinct from hIL-3. Therefore, we attempted to characterize the factor(s) by Sephadex column chromatography. The material responsible for colony stimulation and mast cell growth were distinct. Fig. 3 demonstrates the results from one of three different experiments. When colony formation was measured with mouse bone marrow cells, two major peaks of CSF activity were observed, with mol wts of  $\sim 40$  and  $100 \times 10^3$ . In another experiment using concentrated, pooled SF from five RA patients, a third minor peak was observed at a mol wt of  $\sim 25 \times 10^3$  (data not shown).

The MCGF was considerably smaller, with a mol wt of  $\sim 6 \times 10^3$  (see Fig. 3 B). This activity was heat and acid stable and was resistant to trypsin digestion (data not shown). As suggested by the biphasic dose responses in Fig. 2 B, high molecular weight inhibitors of mIL-3 (see Fig. 3 C) and hMCGF (data not shown) were also present in RA SF. In this experiment, known amounts of mIL-3 or MCGF were added to mast cells in the presence of various column fractions. Inhibitory activity was present in the high molecular weight fractions. It is not known whether this

Colony/10 <sup>5</sup> cells*
0
110
0
34
34 ± 24
21 ± 5
0
0
0

ber of colonies per

1 CSF-1 were active. Five re positive (see Table II). th. Three of the five RA SF and all were positive. or >2 wk. Some disease three psoriatic SF tested n RA fluid cultures and gh some mixed colonies nally identified. Because be made locally. In sup- n samples were negative s had high CSF activity

ile these studies were in it product was produced. ast cells or murine bone rs detected in the mast n hrIL-3. Therefore, we n chromatography. The owth were distinct. Fig. eriments. When colony wo major peaks of CSF In another experiment ird minor peak was ob-

6 × 10<sup>3</sup> (see Fig. 3 B). trypsin digestion (data ig. 2 B, high molecular a not shown) were also mL-3 or MCGF were ons. Inhibitory activity or known whether this

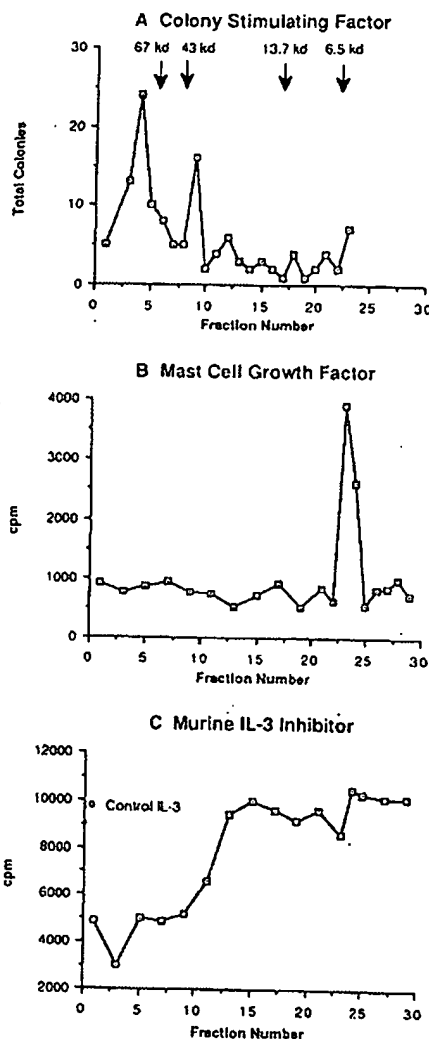


FIGURE 3. Fractionation of RA SF. 2 ml of SF were concentrated fourfold and fractionated by Sephadex G-100 column chromatography. Size standards at top are aligned for all three assays illustrated. (A) CSF. SF fractions (20% by volume) were added to duplicate cultures of murine nonadherent bone marrow cells. Colony count represents sum of colonies after 10 d. (B) MCGF. Mast cell proliferation assay performed as in Fig. 2 using 1:16 dilution of each fraction. (C) mL-3 inhibitor. Mast cell proliferation assay performed as in Fig. 2. In each well, WEHI supernatant (1:8 dilution) was added along with 1:16 dilution of SF fractions.

is due to a specific inhibitor or a toxic constituent of SF, but it is probably the reason that high concentrations of SF were less active than dilute SF in the mast cell assay.

**Identification of the CSF Present in the Rheumatoid Joint.** The predominance of macrophage colonies and the lack of species specificity suggested that the primary CSF in joint effusions detected in the murine assay is CSF-1. Furthermore, CSF-1 has similar molecular weights ( $36-52 \times 10^3$ ,  $70-90 \times 10^3$ ) to the CSF in SF ( $40$  and  $100 \times 10^3$ ). These different forms of CSF-1 are due to variable mRNA splicing and glycosylation (13). To confirm the presence of CSF-1, a specific RIA was used to mea-

sure CSF-1 levels in two ST supernatants and two RA SF samples. The RIA was positive in each case and correlated with the results of the bioassay (see Table III). To positively identify the CSF detected in the murine bone marrow assay as CSF-1, ST supernatants were preincubated with a neutralizing mAb to CSF-1 (14) and were subsequently assayed for residual CSF activity (see Table IV). The majority of the CSF activity was neutralized in two of the three samples tested (the colony count for ST 25 was too low to determine a neutralizing effect accurately). The antibody alone had no effect on murine bone marrow cells and did not inhibit colony formation induced by murine IL-3 (data not shown). Finally, RA synovial RNA was assayed for CSF-1 mRNA using a ribonuclease protection assay. Four of five tissues assayed contained CSF-1 transcript (Fig. 4 shows two positive tissues).

*Human Rheumatoid Synovium Does Not Contain Detectable IL-3 mRNA.* The growth factor bioassays used in these experiments detected CSF-1 and a low molecular weight MCGF. Because no immunoassays or bioassays are available that are specific for human IL-3, we attempted to measure IL-3 mRNA in RA ST cells using a sensitive and specific ribonuclease protection assay. As a positive control, the tissues were first assayed for IL-2-R transcript (see Fig. 5 A). The appropriate 170-base fragment was protected by synovial RNA, indicating the presence of IL-2-R mRNA. Fig. 5 B shows several of the same tissues assayed for IL-3 mRNA. In this case, the 190-base IL-3 probe fragment protected by stimulated Jurkat cells (a T cell line) or activated normal PBMC (data not shown) was not protected in any of the ST assays. Therefore, IL-3 mRNA was not detected in RA synovium (<1-10 messages per cell [10]).

### Discussion

A conventional view of rheumatoid synovitis implicates activated inflammatory cells that indiscriminately secrete a variety of cytokines (1). T cell products in particular are thought to be pivotal in this cascade. However, when levels of cytokines are actually measured, the cytokine milieu is not as complex as initially perceived; instead a restricted pattern of lymphokine production emerges. For instance, IL-3 (or at least its mRNA) is undetectable. IFN- $\gamma$ , a plentiful constituent in supernatants of T cells stimulated with antigens or lectins, is virtually absent from ST and SF (3, 15). The amount of IL-2 in the rheumatoid joint is controversial; using bioassays some investigators have observed IL-2-like activity (16-18), while others have not (19-21). This discrepancy is likely due to non-IL-2 cell growth factors present in the joint. Using an antibody to the IL-2-R and a specific immunoassay, we ob-

TABLE III  
CSF-1 Radioimmunoassay

Sample	CSF-1 (ng/ml)	Colonies/10 <sup>5</sup> cells*
Medium	<1.5	0
SF JB†	25	120
SF XS	6.3	ND
ST RB	2.3	30
ST SW	1.6	20

\* Colonies counted after 10 d.

† JB, XS, RB, SW: initials of patients.

serv  
has  
not  
T  
inhi  
a bi  
IL-2  
cent  
is u  
the i  
imp  
is at  
form  
dem  
a re  
(23)  
the l  
state  
In  
tivity  
ST s  
sent



implies. The RIA was assay (see Table III). arrow assay as CSF-1, o CSF-1 (14) and were 1. The majority of the ted (the colony count rately). The antibody inhibit colony forma- ynovial RNA was as- y. Four of five tissues ve tissues).

mRNA. The growth low molecular weight : that are specific for cells using a sensitive trol, the tissues were ite 170-base fragment -2-R mRNA. Fig. 5 In this case, the 190- (a T cell line) or acti- ny of the ST assays. -10 messages per cell

ivated inflammatory ll products in partic- n levels of cytokines s initially perceived; 1. For instance, IL-3 stituent in superna- absent from ST and versial; using bioas- ), while others have owth factors present mmunoassay, we ob-

TABLE IV  
Effect of Antibody to CSF-1 on Synovial Tissue Supernatant CSF

Sample	Colonies/10 <sup>5</sup> cells*
CSF-1 50 U/ml	64
CSF-1 + anti-CSF-1†	20
ST Z	53
ST Z + anti-CSF-1	13
ST SW	51
ST SW + anti-CSF-1	15
ST 25	14
ST 25 + anti-CSF-1	9

\* Mean number of colonies in duplicate cultures minus mean number of colonies in control dishes. Anti-CSF-1 antibody alone did not affect control dish colony formation and did not affect mIL-3-supported colony formation.

† Samples were preincubated with antibody (100 U/ml neutralizing titer) for 60 min at 4°C before adding culture.

served a CTLL stimulating activity in the joint distinct from IL-2. This activity has not yet been characterized, but is unlikely to be IL-4 because murine cells do not respond to the human factor.

There are two caveats regarding the negative results in the IL-2 assays. First, an inhibitor of IL-2 has been demonstrated in RA SF (22). This could interfere with a bioassay, but the inhibitor only diminishes the response of the indicator cells to IL-2 by ~50% instead of eliminating it entirely. Also, it is only present in low concentrations in SF. Because our CTLL assay detects as little as 1 pg/ml of IL-2, it is unlikely that significant levels of IL-2 would be masked by this inhibitor. Also, the immunoassay confirms the results of the bioassay. A second and potentially more important possibility is that high levels of IL-2 are produced but the lymphokine is absorbed by activated cells. This could not be ruled out by the experiments performed in this study, but in situ immunofluorescence studies of RA synovium has demonstrated little IL-2, consistent with our observations (15). On the other hand, a recent study reported finding IL-2 mRNA in SF mononuclear cells and ST cells (23), but it is not known whether these cells actively secreted IL-2. Regardless of the fate of IL-2 that is secreted into the joint, we have established that the steady-state levels of IL-2 in SF are negligible.

In contrast to the conspicuous absence of IFN- $\gamma$ , IL-2, and IL-3, several new activities were identified in joint tissues and effusions. The majority of RA SF and ST supernatants contain a low molecular weight MCGF. The MCGF may represent another member of a class of small immunomodulating peptides, which in-

s/10<sup>5</sup> cells\*  
0  
120  
ND  
30  
20

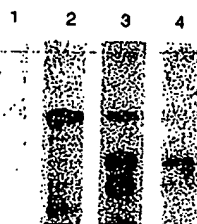


FIGURE 4. CSF-1 mRNA is present in RA synovial tissue. The full-length probe is 311 bases and CSF-1 mRNA protects a 210-base fragment from RNase digestion. Lane 1, unstimulated U937 RNA (negative control); lane 2, PMA/ionophore-stimulated U937 (positive control); lane 3, ST 5; lane 4, ST 6.

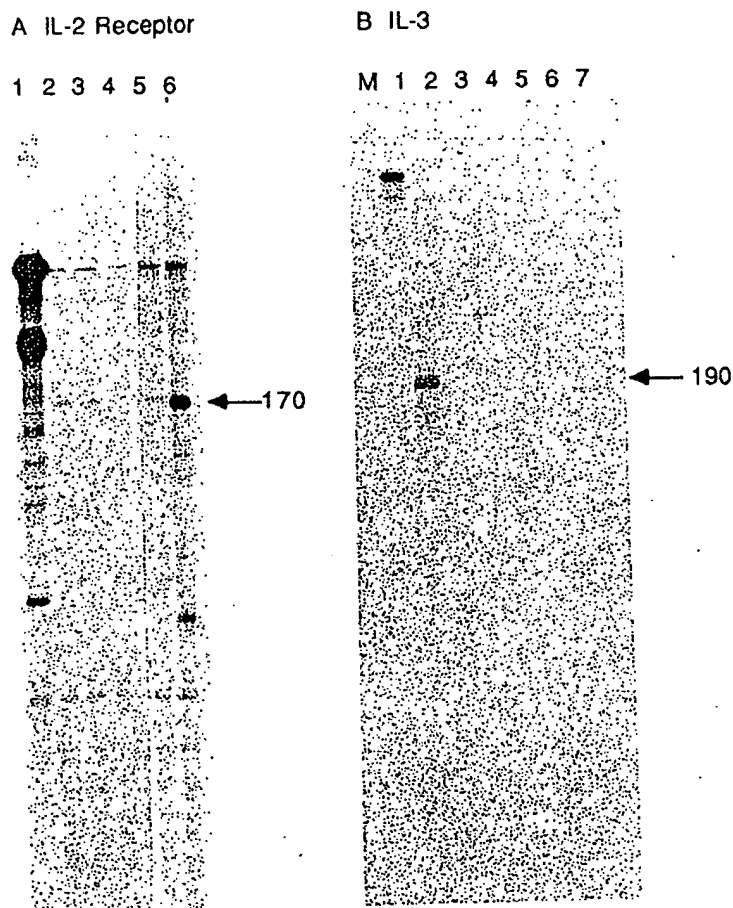


FIGURE 5. Ribonuclease protection assays of ST RNA. RNA was purified from digested ST (see Materials and Methods). (A) IL-2-R mRNA is present in RA ST cells. IL-2-R probe produces a protected fragment of ~170 bases. Lane 1, IL-2-R probe; lane 2, ST 1; lane 3, ST 2; lane 4, ST 2 stimulated with 1  $\mu$ g/ml PHA for 3 d; lane 5, ST 3; lane 6, normal PBM stimulated with 1  $\mu$ g/ml PHA for 1 d. (B) IL-3 mRNA is not detected in RA ST cells. M, size markers; lane 1, IL-3 probe; lane 2, stimulated Jurkat cells; lane 3, ST 1; lane 4, ST 2; lane 5, ST 2 stimulated with PHA for 3 d; lane 6, ST 3; lane 7, ST 4.

cludes CTAPs and neutrophil migration inhibitory factors. The presence of MCGF could explain the local accumulation of mast cells in rheumatoid synovium and SF. Mast cells are a rich source of proteases and inflammatory mediators, like histamine, and may play a role in acute and chronic synovitis. An inhibitor of the MCGF was also identified in SF. Local inhibition of MCGF by this factor may represent a negative feedback loop similar to those previously described for IL-1, IL-2, and IFN- $\gamma$  (3, 22, 24).

Infla  
we rec  
stein, F  
GM-C  
in this  
to loca  
stood,  
tion fr  
tory fu  
in mu  
There  
for tri  
To c  
natani  
source  
as wel  
cell, o  
of the  
in RA  
vial T  
(33).  
mRN  
mitog  
activa  
(33, 3  
after  
is im  
suppi

Be  
synor  
SF au  
line,  
conti  
to IL  
an E  
assay  
In th  
329  
of S  
 $\pm$  1  
of fi  
non.  
nies  
is n  
IL-3

Inflammatory joint fluids also contain colony-stimulating activity. For instance, we recently demonstrated GM-CSF in inflammatory SF (Xu, W. D., G. S. Firestein, R. Tactile, K. Kaushansky, and N. J. Zvaifler, submitted for publication). Human GM-CSF is species specific, however, and can not account for the CSF identified in this study. Using murine bone marrow cells the majority of that activity is due to locally produced CSF-1. The role of CSFs in the rheumatoid process is not understood, but their spectrum of activity extends beyond the induction of colony formation from immature precursors, since many of these factors have other immunomodulatory functions. GM-CSF, for example, increases both IL-1 secretion and Ia expression in murine macrophages (25) and CSF-1 increases macrophage TNF production (26). Therefore, combinations of CSFs may either directly activate APCs or prime them for triggering by other cytokines, thus enhancing synovitis.

To date, most of the mediators identified in synovial fluid or synovial tissue supernatants appear to be produced by non-T cells. Monocytes and macrophages are likely sources of TNF (27), CSF-1 (28), GM-CSF (29), IL-1 (30), and IL-1 inhibitors (24), as well as prostaglandins (31) and collagenase (32). Hence, macrophage, endothelial cell, or synoviocyte activation seems to be crucial to the immunological reactivity of the rheumatoid synovium. When one views the data on synovial ultrastructure in RA from this perspective, the evidence for T cell activation is limited. Most synovial T cells are small and quiescent in appearance, expressing very little surface Ia (33). Although IL-2-R are present on some cells, the degree of expression at the mRNA level is comparable to that of resting peripheral blood T cells rather than mitogen-stimulated cells (our unpublished data). This is in sharp contrast to the activated appearance of macrophages, which express high levels of membrane Ia (33, 34). Although indirect evidence (such as the observation that RA may improve after thoracic duct drainage or total nodal irradiation) suggests that T cell activation is important in rheumatoid synovitis, the cytokine profile of SF and inflamed ST supports the notion that macrophage activation may be a primary event in RA.

### Summary

Because previous studies showed low levels of IFN- $\gamma$  in rheumatoid arthritis (RA) synovial fluid (SF) and synovial tissue (ST) explant supernatants, we assayed RA SF and ST for IL-2 and IL-3-like activity. Using an IL-2 dependent murine CTLL line, 6 of 14 RA SF caused increased thymidine uptake (greater than three times control). The activity was distinct from IL-2 because it was not blocked by antibody to IL-2-R. In addition, IL-2 was not detected (<50 pg/ml) in 16 joint samples using an ELISA. Multi-colony-stimulating factor (CSF) activity was measured using two assays that can detect murine IL-3 (mast cell proliferation, and bone marrow CSF). In the mast cell assay, [ $^3$ H]TdR uptake was  $493 \pm 67$  cpm for medium,  $2,910 \pm 329$  cpm in the presence of RA SF ( $p < 0.001$ ),  $1,246 \pm 156$  cpm in the presence of SF from patients with seronegative spondyloarthropathies ( $p < 0.001$ ), and  $736 \pm 100$  cpm in the presence of osteoarthritis SF ( $p > 0.1$ ). In the CSF assay, four of five RA SF and five of five RA ST induced colony formation from bone marrow nonadherent cells. Macrophage colonies were most common, although mixed colonies and granulocytes were occasionally observed. The multi-CSF activity in RA is not due to IL-3 since human rIL-3 was not active in either murine assay, and IL-3 mRNA was not detected in RA synovium. Sephadex column chromatography

from digested ST  
R probe produces  
lane 3, ST 2; lane  
PBM stimulated  
M, size markers;  
2; lane 5, ST 2

presence of MCGF  
synovium and SF.  
factors, like histat-  
tor of the MCGF  
or may represent  
IL-1, IL-2, and

of RA SF revealed that the mast cell growth factor ( $\sim 6 \times 10^3$  mol wt) and the CSF ( $\sim 40$  and  $100 \times 10^3$  mol wt) are distinct. The colony-stimulating aspect of the "IL-3-like" activity in RA SF is likely due to CSF-1 because it is the appropriate mol wt and because the activity was neutralized by specific anti-CSF-1 antibody. Finally, an RIA detected 1.6–25 ng/ml of CSF-1 in RA SF and ST and CSF-1 mRNA was detected in four of five RA synovial tissue samples tested.

The authors thank Ray Breen and Julie Laxer for technical assistance and Denise Smith for secretarial assistance. Useful discussions with Richard Maki, Ph.D. (La Jolla Cancer Research Foundation) and Peter Ralph, Ph.D. (Cetus Corp.) are acknowledged. Dr. R. Helen Beck generously provided anti-CSF-1 antibody.

Received for publication 3 May 1988 and in revised form 8 August 1988.

### References

1. Cush, J. J., and P. E. Lipsky. 1987. The immunopathogenesis of rheumatoid arthritis: the role of cytokines in chronic inflammation. *Clin. Aspects Autoimmunity* 1:2.
2. Cavender, D., D. Haskard, C.-L. Yu, T. Iguchi, P. Miossec, N. Oppenheimer-Marks, and M. Ziff. 1987. Pathways to chronic inflammation in rheumatoid synovitis. *Fed. Proc.* 46:113.
3. Firestein, G. S., and N. J. Zvaifler. 1987. Peripheral blood and synovial fluid monocyte activation in inflammatory arthritis. II. Low levels of synovial fluid and synovial tissue interferon suggest that gamma-interferon is not the primary macrophage activating factor. *Arthritis Rheum.* 30:864.
4. Burmester, G. R., D. T. Y. Yu, A. M. Irani, H. G. Kunkel, and R. J. Winchester. 1981. Ia+ T cells in synovial fluid and tissues of patients with rheumatoid arthritis. *Arthritis Rheum.* 24:1370.
5. Burmester, G. R., B. Jahn, M. Gramatzki, J. Zacher, and J. R. Kalden. 1984. Activated T cells in vivo and in vitro: divergence in expression of Tac and Ia antigens in the non-blastoid small T cells of inflammation and normal T cells activated in vitro. *J. Immunol.* 133:1230.
6. Hogg, N., D. G. Palmer, and P. A. Revell. 1985. Mononuclear phagocytes of normal and rheumatoid synovial membrane identified by monoclonal antibodies. *Immunology* 56:673.
7. Ropes, M. W., G. A. Bennett, S. Cobb, R. Jacox, and R. A. Jessar. 1958. 1958 revision of diagnostic criteria for rheumatoid arthritis. *Bull. Rheum. Dis.* 9:175.
8. Gillis, S., M. Ferm, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.
9. Lowenthal, J. W., P. Cortesy, C. Tougne, R. Lees, H. R. MacDonald, and M. Nabholz. 1985. High and low affinity IL-2 receptors: analysis by IL-2 dissociation rate and reactivity with monoclonal anti-receptor antibody PC61. *J. Immunol.* 135:3988.
10. Firestein, G. S., S. M. Gardner, and W. D. Roeder. 1987. Quantitative molecular hybridization with unfractionated, solubilized cells during RNA probes and polyacrylamide gel electrophoresis. *Anal. Biochem.* 167:381.
11. Ihle, J. N. 1985. Immunological regulation of hematopoietic stem cell differentiation by interleukin-3. *Year Immunol.* 107-117.
12. Yang, Y. C., A. B. Ciarletta, P. A. Temple, M. P. Chung, S. Kovacic, J. S. Witek-Giannotti, A. C. Leary, R. Kriz, R. E. Donahue, G. G. Wong, and S. C. Clark. 1986. Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell* 47:3.
13. Wong, G. G., P. A. Temple, A. C. Leary, J. S. Witek-Giannotti, Y. C. Yang, A. B. Ciarletta,

ol wt) and the CSF  
aspect of the "IL-  
e appropriate mol  
l antibody. Finally,  
CSF-1 mRNA was

e and Denise Smith  
La Jolla Cancer Re-  
dged. Dr. R. Halen

heumatoid arthritis:  
munology. 1:2.  
ppenheimer-Marks,  
l synovitis. *Fed. Proc.*

ovial fluid monocyte  
l and synovial tissue  
age activating factor.

J. Winchester. 1981.  
id arthritis. *Arthritis*

den. 1984. Activated  
antigens in the non-  
in vitro. *J. Immunol.*

macrophages of normal  
bodies. *Immunology.*

1958. 1958 revision  
:175.

factor: parameters  
. 120:2027.

d, and M. Nabholz.  
ation rate and reac-  
35:3988.

ative molecular hy-  
and polyacrylamide

cell differentiation

S. Witek-Giannotti,  
lark. 1986. Human  
matopoietic growth

ang, A. B. Ciarletta,

- M. Chung, P. Murtha, R. Kriz, R. J. Kaufman, C. R. Ferenz, B. S. Sibley, K. J. Turner, R. M. Hewic, S. C. Clark, N. Yanai, H. Yokota, M. Yamada, M. Saito, K. Motoyoshi, and F. Takak. 1987. Human CSF-1: molecular cloning and expression of 4-kb cDNA encoding the human urinary protein. *Science (Wash. DC)*. 235:1504.
14. Halen Beck, R., P. J. Shadel, P.-J. Lee, M. T. Lee, and K. Koths. 1988. Purification and characterization of recombinant human macrophage colony-stimulating factor and generation of a neutralizing antibody useful for Western analysis. *Biotechnol. J.* 8:44.
15. Husby, G., and R. C. William. 1985. Immunohistochemical studies of interleukin 2 and gamma interferon in rheumatoid arthritis. *Arthritis Rheum.* 28:174.
16. Lemm, G., and H. Warnatz. 1986. Evidence for enhanced interleukin 2 (IL-2) secretion and IL-2 receptor presentation by synovial fluid lymphocytes in rheumatoid arthritis. *Clin. Exp. Immunol.* 64:71.
17. Nouri, A. M. E., G. S. Panayi, and S. M. Goodman. 1984. Cytokines and the chronic inflammation of rheumatoid disease. II. The presence of interleukin-2 in synovial fluids. *Clin. Exp. Immunol.* 58:402.
18. Wilkins, J. A., R. J. Warrington, S. L. Sigurdson, and J. Rutherford. 1983. The demonstration of an interleukin 2-like activity in the synovial fluids of rheumatoid arthritis. *J. Rheumatol.* 10:109.
19. Egeland, T., and H. Lund. 1987. Immunoregulatory lymphokines in rheumatoid joints. I. Search for interleukin 2 in synovial fluid. *Scand. J. Immunol.* 25:101.
20. Fontana, A., H. Hengartner, E. Weber, K. Fehr, P. J. Grop, and G. Cohen. 1982. Interleukin 1 activity in the synovial fluid of patients with rheumatoid arthritis. *Rheumatol. Int.* 2:49.
21. Wood, D. D., E. J. Ihrie, C. A. Dinarello, and P. L. Cohen. 1983. Isolation of an interleukin-1-like factor from human joint effusions. *Arthritis Rheum.* 26:975.
22. Miossec, P., T. Kashiwado, and M. Ziff. 1987. Inhibitor of IL-2 in rheumatoid synovial fluid. *Arthritis Rheum.* 30:121.
23. Buchan, G., K. Barrett, T. Fujita, T. Taniguchi, R. Maini, and M. Feldmann. 1988. Detection of activated T cell products in the rheumatoid joint using cDNA probes to Interleukin-2 (IL-2) IL-2 receptor and IFN-gamma. *Clin. Exp. Immunol.* 71:295.
24. Lotz, M., C. D. Tsoukas, C. A. Robinson, C. A. Dinarello, D. A. Carson, and J. H. Vaughan. 1986. Basis for defective responses of rheumatoid arthritis synovial fluid lymphocytes to anti-CD3 (T3) antibody. *J. Clin. Invest.* 78:713.
25. Morrissey, P. J., L. Bressler, L. S. Park, A. Alpert, and S. Gillis. 1987. Granulocyte-macrophage colony-stimulating factor augments the primary antibody response by enhancing the function of antigen-presenting cells. *J. Immunol.* 139:1113.
26. Warren, M. K., and P. Ralph. 1986. Macrophage growth factor CSF-1 stimulates human monocyte production of interferon, tumor necrosis factor, and colony stimulating activity. *J. Immunol.* 137:2281.
27. Old, L. J. 1985. Tumor necrosis factor (TNF). *Science (Wash. DC)*. 230:630.
28. Rambaldi, A., D. C. Young, and J. D. Griffin. 1987. Expression of M-CSF (CSF-1) gene by human monocytes. *Blood.* 69:1409.
29. Piacibello, W., L. Lu, M. Wachter, B. Rubin, and H. E. Broxmeyer. 1985. Release of granulocyte-macrophage colony stimulating factors from major histocompatibility complex class II antigen-positive monocytes is enhanced by human gamma interferon. *Blood.* 66:1343.
30. Dinarello, C. A. 1985. An update on human interleukin-1: From molecular biology to clinical relevance. *J. Clin. Immunol.* 5:287.
31. Scott, W. A., N. A. Pawlowski, H. W. Murray, M. Andreach, J. Zrike, and Z. A. Cohn. 1982. Regulation of arachidonic acid metabolism by macrophage activation. *J. Exp. Med.* 155:1148.

32. Wahl, I. M., S. M. Wahl, S. E. Mergenhagen, and G. R. Martin. 1975. Collagenase production by lymphokine-activated macrophages. *Science (Wash. DC)*. 187:261.
33. Iguchi, T., M. Kurosaka, and M. Ziff. 1985. Electron microscopic study of HLA-DR in monocyte/macrophage staining cells in the rheumatoid synovial membrane. *Arthritis Rheum.* 29:600.
34. Firestein, G. S., and N. J. Zvaifler. 1987. Peripheral blood and synovial fluid monocyte activation in inflammatory arthritis. I. A cytofluorographic study of monocyte differentiation antigens and class II antigens and their regulation by gamma-interferon. *Arthritis Rheum.* 30:857.

G)  
F  
I

h

(  
has  
in )  
ant  
the  
(4,  
the  
tigr  
7).  
im  
hav  
spe  
of i  
(11,  
res  
hui  
tigr  
inf  
inf  
to  
I  
of t  
tive  
sio  
to  
Th

M.  
Pat  
Me  
vinc  
PER  
vira

The

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**